(54) Title: NOVEL DRUG DELIVERY SYSTEMS FOR PROTEINS AND PEPTIDES USING ALBUMIN AS A CARRIER MOLECULE

(57) Abstract

    A drug delivery system for proteins and peptides using albumin as a carrier molecule. The drug is bound noncovalently to albumin by means of an attached apolar substituent, preferably a long chain fatty acid derivative.

NOVEL DRUG DELIVERY SYSTEMS FOR PROTEINS AND PEPTIDES USING
ALBUMIN AS A CARRIER MOLECULE
BACKGROUND OF THE INVENTION

This invention relates to a novel drug carrier
system to enhance the efficacy of peptides and
proteins used as therapeutic agents. Improvements
in the chemical methods for peptide synthesis and
the use of recombinant DNA techniques to produce
larger polypeptides and proteins have greatly
expanded the therapeutic uses of these drugs.
Representative applications include the use of
superoxide dismutase (SOD) as an anti-inflammatory
and anti-ischemic agent, the soluble CD4 protein for
the treatment of AIDS, polypeptide hormones such as
human insulin, gamma-interferon and other
lymphokines for the treatment of infectious diseases
and certain types of cancer, growth factors such as
growth hormone and erythropoeitin which is useful
in the treatment of anemia, and tissue plasminogen
activator useful as a thrombolytic agent. A further
advantage of the new methods of synthesis of these
peptides and proteins is that problems of immuno-
genicity, which often arise if the molecule is
isolated from an animal source, can be avoided by
producing the native human protein.

However, one common limitation in the use of
proteins and peptides as drugs is that they are
rapidly metabolized or excreted in the urine. The
renal threshold for filtration of molecules
dissolved in plasma is in the range of 50,000 to
60,000 daltons. Smaller molecules are very rapidly
filtered from the circulation by the kidneys. For
example, SOD with a molecular weight of only 32,000
daltons has a half-life in plasma of only about 6
minutes. Hemoglobin normally a tetramer of two
alpha and two beta polypeptide chains can dissociate
into alpha-beta dimers which also have a molecular

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weight of just 32,000. At typical therapeutic
concentrations when acellular hemoglobin is used as
a blood replacement, about 3 to 5 percent of the
protein exists as dimers. As in the case of SOD,
the dimers are cleared from the circulation with a
half-life on the order of minutes. As the
concentration of dimers becomes depleted the
equilibrium

$$\alpha_2\beta_2 \;\rightleftharpoons\; 2\,\alpha\beta \quad (1)$$
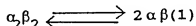
becomes progressively shifted to the right. The
newly formed dimers are likewise excreted. This
process continues until all of the hemoglobin is
eliminated. The overall half-life of hemoglobin in
the circulation is about 90 minutes. Not only does
this compromise its function as a blood substitute,
but the massive dumping of protein in the urine also
poses the risk of renal injury.

    Several approaches have been taken to prolong
the intra-vascular retention time of such molecules,
all of which involve extensive covalent modifi-
cation of the protein. A variety of nonspecific
cross-linking agents have been employed to
polymerized hemoglobin to produce higher molecular
weight species (see for example Bonsen, U.S. Patent
4,001,401). Most commonly, glutaraldehyde has been
used which reacts more or less randomly with the
amino groups of the 44 lysine residues and 4 amino-
termini of the hemoglobin molecule. This results
in a polydisperse mixture which appears at best to
be of limited clinical utility. Alternatively,
hemoglobin and other proteins of therapeutic
interest have been covalently cross-linked to a
macromolecular carrier such as dextran or albumin,
again using random cross-linking reagents like
glutaraldehyde (Wong, J.T. (1988) Biomaterials,

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Artificial Cells, and Artificial Organs 16, 237-
245; Poznansky, M.J. (1988) Methods in Enzymology
137, Academic Press, New York, pp. 566-574). A
third approach which has been taken to increase the
effective size of polypeptide and protein drugs,
and thereby prevent their rapid renal excretion, is
to decorate the molecule with multiple copies of a
lower molecular weight substance. Polyethylene
glycol in the molecular weight range of 2,000 to
10,000 daltons has been used most frequently for
this purpose, (Abuchowski, A. et al., (1977) Journal
of Biological Chemistry 252, 3578-3581, 3582-3586;
Pyatak, P.S. et al. (1980) Research Communications
in Chemical Pathology and Pharmacology 29, 113-
127).

     The extensive modification of the protein that
results using all three of these methods invariably
leads to a very heterogenous mixture of products.
The composition can be characterized only by certain
average properties making batch to batch uniformity
difficult to insure. Extensive modification of the
protein or peptide can also lead to a decrease or
loss of activity; and especially in the case of
cross-linking agents, problems of antigenicity can
arise. To date, there are no protein or peptide
cross-linked conjugates that are in use clinically;
and there are only a few examples in which
decoration with polyethylene glycol has lead to the
development of a useful therapeutic agent, despite
the introduction of this approach nearly 15 years
ago. Thus, while some progress has been made, there
remains a continuing need for improved delivery
systems for protein and peptide drugs. The primary
objective of the present invention is to fulfill
that need.

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A yet further objective of the present invention is to provide a drug carrier system for proteins and peptides in which such molecules are modified at a unique or a limited number of sites with an apolar (hydrophobic) substituent to serve as a bridging group to mediate binding of the drug noncovalently to albumin.

A yet further objective of the invention is to provide reagents which can be used to selectively modify peptides and proteins with fatty acid derivatives.

A yet still further objective of the present invention is to provide a means of regulating the activity of peptides and proteins used therapeutically by competition with free fatty acids for binding sites on albumin, resulting in displacement of the drug.

SUMMARY OF THE INVENTION

A new drug delivery system for peptides and proteins wherein such molecules are modified with apolar substituents to promote noncovalent binding to albumin, which in turn serves as a macromolecular carrier for the drug within the circulation. In the most preferred embodiment of the invention the protein is modified at a single or limited number of sites with a long chain fatty acid derivative. Novel protein modifying agents to attach such groups are provided. A method for regulating the activity of such drugs based on competition for binding sites on albumin with free fatty acids is also presented.

DETAILED DESCRIPTION OF THE INVENTION

Albumin is the most abundant plasma protein and has multiple binding sites for apolar molecules. In

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the present invention albumin is used as a carrier
molecule for the transport of peptide and protein
drugs within the circulation. The invention is
especially useful for polypeptides below a molecular
weight of about 60,000 which normally would be
rapidly excreted in the urine. Biding of the
protein to albumin is mediated by an attached
derivative. Long chain fatty acid derivatives are
the preferred bridging groups to provide high
binding affinity.

The molecular weight of the albumin is 66,000
daltons. Normally very little of the protein (less
than 100 mg/day) is excreted in the urine. Albumin
plays an important role physiologically in the
transport of free fatty acids within the circula-
tion. There are at least 10 sites on the protein at
which fatty acids can bind. The two highest
affinity binding sites are selective for fatty
acids. The remaining binding sites can accommodate
a variety of apolar molecules, particularly organic
anions. The association constant for the binding
of long chain fatty acids such as palmitate (C_{16})
and stearate (C_{18}) at the high affinity sites is on
the order of 10^8 M^{-1}. There are 3 to 5 secondary
sites for which the binding constant is about
10^6 M^{-1}, still relatively large. The binding energy
is due primarily to hydrophobic interactions. A
smaller, but significant contribution is due to
electrostatic interactions between the carboxylate
group and a surface lysine residue of the protein
adjacent to the apolar binding pocket. The
carboxylate group can be replaced with other
negatively charged substituents, including but not
limited to, phosphates, phosphonates, sulfates and
sulfonates without appreciably affecting the binding

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affinity. The molar ratio of free fatty acids to
albumin in the blood is typically about 0.6. At the
highest plasma concentration of free fatty acids
observed physiologically, which occurs during
strenuous exercise, the molar ratio is 4. Thus
under all conditions there are vacant binding sites
on the protein with affinities for free fatty acids
of at least 10^6 M^{-1}. For further discussion of the
structure and function of albumin see Spector, A.A.
(1986) in Biochemistry and Biology of Plasma
Lipoproteins (Scanu, A.M. and Spector, A.A., eds.)
Marcel Dekker, Inc., New York, pp. 247-279, which is
incorporated herein by reference.

     A variety of apolar molecules can be used as
the bridging group to promote the binding of
peptides and proteins to albumin. Organic anions
are preferred. The most preferred bridging groups
are long chain fatty acid derivatives. The length
of the hydrocarbon chain can vary from about 10 to
about 24 carbon atoms, preferably 16 to 20 carbon
atoms. Carbon-carbon double bonds, such as in
derivatives of oleic acid, carbon-carbon triple
bonds, and heteroatoms uncharged at physiological
pH, including but not limited to oxygen and sulfur,
can also be incorporated into the chain. A number
of different negatively charged groups may be
introduced into the molecule to enhance the binding
affinity to albumin, including carboxylates,
phosphates, phosphonates, sulfates and sulfonates.
Functional groups which may be incorporated into the
reagent to provide a means of attachment to the
protein include: (1) activated carboxylic acid
derivatives such as anhydrides, N-hydroxy-
succinimide esters, or phenylesters; (2) alkylating
agents such as alkyl halides, alpha-halo carboxylic

acids and amides or N-substituted maleimides; and
(3) carbonyl groups, aldehydes and ketones, which
may be used to link the compound to amino groups of
the protein by reductive alkylation. For further
discussion of the reactive groups which may be used
to covalently modify different amino acid residues
of proteins and peptides see Lundblad, R.L., and
Noyes, C.M. (1984) Chemical Reagents for Protein
Modification, CRC Press, Boca Raton, Florida, which
is incorporated herein by reference.

     In the preferred practice of the invention the
attachment of the apolar group is restricted to a
unique site or a limited number of positions on the
protein or peptide. This makes it possible to
produce a specific derivative having well defined
and desirable properties, in contrast to the random
mixture of reaction products which results from
cross-linking proteins and peptides to macromolecu-
lar carriers. The reagent may be directed to a
specific region of the molecule on the basis of
noncovalent binding interactions at the site.

     Example 2 below illustrates the use of such an
affinity reagent to modify hemoglobin with a long
chain fatty acid derivative selectively at the 2,3-
diphosphoglycerate binding pocket located at the
interface between the beta chains. Alternatively,
the sites of modification may be restricted by using
reagents that react at the SH group of cysteine
residues. Cysteine is uniquely reactive among the
amino acid residues naturally found in proteins and
can be readily targeted with a number of different
alkylating agents such as alpha-halo carboxylic
acids and amides and maleimide derivatives (see
Lundblad and Noyes, 1984). Moreover, most proteins
have only a limited number of free cysteine

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residues. In hemoglobin there are three: beta 93,
beta 112 and alpha 104 (six in total per tetramer).
Beta 112 and alpha 104 are buried inside the
molecule; only cysteine beta 93 is reactive. In SOD
there are four cysteines per 32,000 molecular weight
dimer; only the two cysteine 111 residues are
reactive. In proteins in which a cysteine residue
is absent, one may be readily introduced using
recombinant DNA techniques. For shorter
polypeptides, synthetic methods can be used to
replace one of the native amino acids within the
sequence with cysteine, or an additional cysteine
residue can be added to the molecule. There are, of
course, certain amino acid residues that are
critical for the activity of any given protein which
cannot be modified, but generally there are a number
of positions at which amino acid substitutions can
be made with relatively little effect on the
properties of the molecule.

Two novel classes of modifying agents have been
developed which can be used to attach fatty acid
derivatives to proteins and peptides:

$$
\overset{(X)}{/\!/\!/\!/\!/\!/\!/\!/\!/\!/\!/\!/\!/\!/\!/}\overset{COO^-}{\underset{/\!/\!/\!/\!/\!/\!/\!/\!/\!/\!/\!/Z}{\diagup}} \tag{I}
$$
$$
\underset{(Y)}{}
$$

and

$$
\overset{(X)}{/\!/\!/\!/\!/\!/\!/\!/\!/\!/\!/\!/\!/\!/}-O-\overset{\overset{O}{\|}}{\underset{\underset{O^-}{|}}{P}}-O-\overset{(Y)}{/\!/\!/\!/\!/\!/\!/\!/Z} \tag{II}
$$

In (I) the negatively charged substituent is provided
by a carboxylate group, in (II) by a phosphate
group. The long chain apolar group (X) which will
insert into the hydrophobic binding pocket of
albumin can vary in length from about 10 to about 24

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atoms, preferably 16 to 20 atoms. Heteroatoms such
as oxygen and sulfur, as well as other apolar
functionalities may be incorporated into the chain.
The spacer arm (Y) through which the fatty acid
derivative is attached to the protein or peptide can
be a simple aliphatic chain, or it may incorporate
polar or charged groups to enhance water solubility.
Aromatic substituents may also be incorporated into
the spacer arm. The functional group (Z) provides
the means of attachment of the reagent to the
protein or peptide. It may be any of the reactive
groups used to modify proteins described above.
Syntheses of representative examples of these two
classes of compounds is described in Example 1.
Example 2 demonstrates the reaction of these
reagents with hemoglobin.

     Given the high concentration of albumin within
plasma (about 0.65 mM) and the multiple number of
available binding sites, an association constant of
only 10^5 M^{-1} will ensure that over 99% of a fatty
acid derivatized protein or peptide will be bound to
albumin. Even the secondary binding sites have
association constants of 10^6 M^{-1} for free fatty
acids. Because the concentration of albumin will
generally be in vast excess over the therapeutic
level of the protein or peptide drug, a 1:1 complex
between albumin and the drug molecule will be
formed; i.e. there will be a negligible fraction of
species in which two or more molecules of the drug
are bound to the same molecule of albumin. One
important exception to this is in the use of
hemoglobin as a blood substitute. In this case, the
plasma concentration of hemoglobin is on the order
of 1 to 2 mM. Moreover, the level of albumin is
depleted due to the loss of blood. Consequently,

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higher molecular weight species in which there are
two or more molecules of hemoglobin attached to a
single molecule of albumin can be formed.

Not only is renal excretion of the protein
bound to albumin blocked, but filtration across
other capillary beds is also limited. This is
particularly true of hemoglobin blood substitutes
bound to albumin due to the higher molecular weight
species that are formed. Binding of the protein to
albumin can also enhance lymphatic return from the
interstitial fluid. Even after cross-linking
hemoglobin intramolecularly to prevent dissociation
of the tetramer the intravascular retention time is
much shorter than that of albumin, despite the fact
that both proteins have nearly the same molecular
weight. The initial rate of egress of the two
proteins from the circulation is, however, nearly
the same. Whereas nearly all of the albumin
filtered is returned to the blood via the lymphat-
ics, very little of the hemoglobin is recovered.

Protein and peptide drugs used in accordance
with the present invention are generally
administered parenterally. Co-administration of
the drug with albumin may be employed but is usually
not required. Binding of the modified protein or
peptide occurs rapidly to the endogenous albumin
within the circulation, or to albumin within the
interstitial fluid if the drug is given subcuta-
neously or intramuscularly. Replacement of albumin
may become necessary in the treatment of severe
hemorrhage when using fatty acid modified hemoglobin
derivatives as blood substitutes. Suitable
pharmaceutical carriers for the modified protein
and polypeptide drugs of the present invention
include but are not limited to physiological

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saline, a mixture of glucose and saline or Ringer's
lactate. Conventional methods for administering
such therapeutic agents are known medical state of
the art, see for example, Avis, K. (1985) in
Remington's Pharmaceutical Sciences (Gennaro, A.R.,
ed.) Mack Publishing Company, Easton, PA, pp. 1518-
1541, which is incorporated herein by reference.

Proteins or peptides modified with apolar
substituents may remain fully active while
associated with albumin. Such is the case for SOD
and hemoglobin derivatives, for example.
Alternatively, only the free polypeptide may retain
activity, in which case the complex with albumin
would provide an inactive depot form of the drug.
Since the vast majority of the protein is bound to
albumin, this provides an effective reservoir to
buffer the free concentration of the drug. As the
free form of the drug becomes metabolized or
excreted, the equilibrium in equation (2) shifts
progressively to the right, i.e. the protein becomes
released from albumin. This maintains the
concentration of the free drug, and hence the level
of activity, in a relatively narrow range between
doses.

$$\text{Albumin Protein} \rightleftharpoons \text{Albumin } + \text{ Protein} \qquad (2)$$
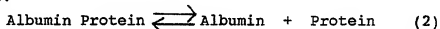
Using albumin as a carrier molecule for protein
and peptide drugs offers further advantages.
Association of the drug with albumin may protect it
from proteolytic or other enzymatic degradation.
Since the binding of the protein to albumin is
reversible free fatty acids or other apolar
molecules can be administered to displace the
protein from the complex by competition for the
available binding sites on albumin. For small
proteins and peptides which in their free form are

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very rapidly excreted in the urine, i.e. on the
order of minutes, this will abruptly terminate the
activity of the agent. This provides a novel means
by which the duration of action of protein and
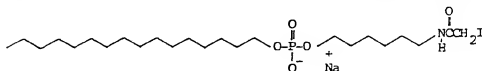peptide drugs can be precisely controlled.

     The present invention also affords a novel
class of long acting insulin derivatives which bind
to albumin by means of an attached apolar group.
Competition with endogenous free fatty acids gives
rise to particularly useful pharmacokinetic
properties for these derivatives. Normally there is
an excess of available binding sites on albumin so
that the modified insulin is almost 100% in the
bound form. In this state, the insulin is inactive.
Regular insulin is given intermittently to maintain
normal metabolic control. If the levels of insulin
administered are inadequate, the concentration of
free fatty acids in the blood increases. This
displaces some of the insulin bound to albumin and
ameliorates the insulin deficiency. Such apolar
modified insulin derivatives are particularly useful
in preventing the complications of diabetic keto-
acidosis, in which case the free fatty acid
concentration can be as high as 3 to 4 mM.

     The following examples are offered to further
illustrate, but not limit, the process, products and
medical techniques of the invention.

                    Example 1
        Synthesis of Novel Fatty Acid Protein and
              Peptide Modifying Agents

Sodium 1-hexadecyl, 6-(2-iodoacetomido)hexyl phosphate ($\underline{5}$)

$$\sim\!\!\sim\!\!\sim\!\!\sim\!\!\sim\!\!\sim\!\!\sim\!\!\sim\!\!\!O-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O^-}{|}}{P}}-O\sim\!\!\sim\!\!\sim\!\!\sim\!\!\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle H}{|}}{N}}CCH_2I$$

                              Na

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This new compound was synthesized by the following 5 step sequence. In the first step 1-hexadecanol (2.42 g, Aldrich) was condensed with 2-cyanoethyl-N,N-diisopropylamino chlorophosphine (2.84 g, American Bionetics) in dry methylene chloride in the presence of 2.58 g of diiso-propylethylamine. The reaction was complete within 10 minutes. The mixture was poured into ethyl acetate and was washed first with saturated sodium bicarbonate, then with brine. The solution was then dried with sodium sulfate and the solvent was evaporated. The crude material was chromatographed over silica gel using 25% ethyl acetate:65% hexanes:10% triethylamine as the solvent to isolate the product: 1-hexadecanyl, 2-cyanoethyl N,N-diisopropylphosphoramidite (1), as a colorless liquid.

Compound 1 (220 mg) was next coupled to N-(p-anisyldiphenylmethyl)-6-aminohexanol (194mg, prepared as described by Connolly, B.A., Nucleic Acids Research (1987) 15, 3131-3139). The reaction was carried out in dry acetonitrile in the presence of tetrazole (2 equivalents) as an activator. After 15 minutes, the reaction mixture was evaporated to dry-ness, resuspended in hexanes and then chromatographed over silica gel. The product: 1-hexadecyl,2-cyanoethyl N-(p-anisyldiphenylmethyl)-6-aminohexyl phosphite (2) was eluted with 84% hexanes:15% ethyl acetate:1% triethylamine as a colorless oil.

Next 2 was oxidized with t-butyl hydroperoxide (2.2 equivalents) to afford the corresponding phosphate (3). The reaction was carried out in methylene chloride for 10 minutes. The solvent was evaporated and the residue chromatographed over silica using 50% ethyl acetate:50% hexanes as the eluant.

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The 2-cyanoethyl group was next removed by refluxing 3 in triethylamine:acetonitrile (1:1) for 5 hours. After removal of the solvents, the p-anisyldiphenylmethyl group was removed by treatment with 80% aqueous acetic acid at room temperature for 3 hours. The acetic acid was then removed by evaporation. The residue was dissolved in water and washed with hexanes to remove p-anisyldiphenylmethyl alcohol. The aqueous layer was then evaporated and the product 1-hexadecyl, 6-aminohexyl phosphate (4) was isolated as a white precipitate by the addition of ethyl ether.

Finally, the title compound 5 was obtained by the reaction of 4 with iodoacetic anhydride. Compound 4 (210 mg) was dissolved in 3.0 ml of 1.0 N NaOH at 10°C. A solution of iodoacetic acid (530 mg) in 3 ml of acetonitrile was then added quickly to the aqueous solution with vortexing. After one minute, the organic layer was separated, dried over sodium sulfate, and evaporated to give a white solid. The product was dissolved in a few drops of chloroform and reprecipitated by the addition of 5 ml of ethyl acetate.

2-((8-[N-(2-(Iodoacetylamino)ethyl)carboxamido]
octyl)thio)hexadecanoic acid (12)
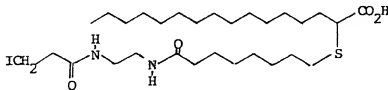
This new fatty acid derivative was synthesized according to the following reaction sequence. First 8-thiooctanoic acid (6) was prepared by refluxing 8-

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bromooctanoic acid (10.65 g) with thiourea (3.65 g)
in ethanol (25 ml) for 7 hours. Ten ml of a 0.1 N
NaOH solution was then added and the reaction mix-
ture was further refluxed for 3.5 hours and then
allowed to cool to room temperature and stand
overnight. After addition of water, the solution was
extracted with ethyl acetate then acidified with 10%
sulfuric acid. The organic layer was then washed
with brine, evaporated to give an oily residue which
was then redissolved in hexanes. A white solid
appeared which was removed by filtration. The
filtrate was evaporated to dryness to give the crude
product. Chromatography over silica gel using 5%
ethyl acetate in hexanes as the eluant afforded the
pure compound.

Methyl 8-thiooctanoate (7) was prepared from
the acid, 6, by esterification with excess ethereal
diazomethane.

t-Butyl 2-bromohexadecanoate (8) was
synthesized from 2-bromohexadecanoic acid (11.4 g) in
dry t-butanol (250 ml) in the presence of
dicyclohexylcarbodiimide (7.5 g, added as a solution
in 50 ml of tetrahydrofuran) and 4-pyrrolidinopyri-
dine (200 mg). The reaction mixture was allowed to
stir for 2 hours at room temperature and then
evaporated to dryness. The residue was heated in
ether (400 ml) and solid oxalic acid (5.0 g) was
introduced. The mixture was filtered and the
filtrate concentrated to dryness under vacuum. The
desired product was purified by recrystallization
from methanol.

t-butyl 2-((8-Carbomethoxyoctyl)thio)hexa-
decanoate (9) was prepared by the reaction of 7 (2.82
g) with 8 (5.8 g) in methanol (65 ml) in the presence
of sodium (0.34 g). The reaction mixture was

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refluxed for 5 hours and then left overnight at room
temperature. After concentration of the reaction
mixture, addition of ice resulted in the
precipitation of 9 as a white solid which was then
purified by recrystallization from methanol.

     Next, a mixture of 9 (0.91 g) and ethylene
diamine (8.0 g) was heated at 85°C for 10 hours. The
solution was then further stirred at room temperature
for 72 hours. Excess ethylene diamine was removed by
heating at 60°C under vacuum. The residue was
diluted with water and extracted exhaustively with
ethyl acetate. The organic layer was washed with
water, dried with sodium sulfate and evaporated to
give t-butyl 2-((8-[N-(2-aminoethyl)carboxamido]-
octyl)thio)hexadecanoate (10) as a yellow oil which
was carried forward without further purification.

     A mixture of 10 (1.64 g) and iodoacetic
anhydride (1.63 g) in 10 ml of tetrahydrofuran was
stirred at room temperature for 10 hours. The
solvent was evaporated under reduced pressure and
the residue was redissolved in ethyl acetate. The
organic layer was washed with 2% sodium bisulfite
then with brine and then evaporated to dryness. The
crude product was recrystallized from aqueous
methanol to give t-butyl 2-((8-[N-(2-(iodoacetyl-
amino)ethyl)carboxamido]octyl)thio)hexadecanoate
(11). M.p. 127-128°C.

     The title compound 12 was prepared by treating
11 with trifluoroacetic acid for 20 hours at room
temperature to remove the t-butyl protecting group.
After removing the trifluoroacetic acid under
reduced pressure, the product was recrystallized
from ethyl acetate. M.p. 101-102°C.

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2-([8-(Carbo(3,5-dicarboxy)phenoxy)octyl]thio)
hexadecanoic acid (15)
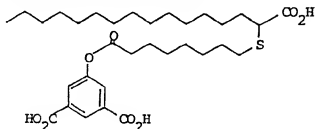
        To a solution of sodium (300 mg) in 25 ml of
absolute ethanol was added 6 (1.24 g) followed by
addition of 8 (2.5 g). The reaction mixture was
refluxed for 8 hours and left overnight at room
temperature. The concentrated reaction mixture was
diluted with ice water and acidified with chilled 1%
acetic acid. Extraction with ethyl acetate followed
by washing of the organic layer with water, drying
over sodium sulfate and evaporation under reduced
pressure afforded t-butyl 2-((8-carboxyoctyl)thio)-
hexadecanoate (13) as a colorless oil.
        To a solution of 13 (0.97 g), 4-pyrrolidino-
pyridine (10 mg) and dibenzyl 5-hydroxyisophthalate
(0.72 g) in 6 ml of tetrahydrofuran was added
dicyclohexylcarbodiimide (0.46 g). The reaction
mixture was stirred for 3.5 days after which 230 mg
of dicyclohexylcarbodiimide was added and the
reaction allowed to proceed for an additional day.
The solvent was removed and 50 ml of ether was added
followed by 260 mg of oxalic acid. The suspension
was stirred for 15 minutes and then filtered. The
filtrate was concentrated to give the desired
product, t-butyl 2-([8-(carbo(3,5-dibenzylcarboxy)-
phenoxy)octyl]thio)hexadecanoate (14) which was
purified by silica gel chromatography.

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for 6 hours over 20% Pd(OH)$_2$/C in cyclohexene:ethanol
(1:1). The resulting compound was then treated with
trifluoroacetic acid at room temperature to remove
the t-butyl group.

3,5-Dicarboxyphenyl palmitate (<u>16</u>)
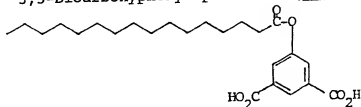
        The title compound was prepared by the reaction
of palmitoyl chloride (1.7 ml) with 5-hydroxyiso-
phthalic acid (1 g) in dry tetrahydrofuran. Both
starting materials were purchased from Aldrich. The
reaction was allowed to proceed for 5 hours at room
temperature after which the solvent was evaporated.
The residue was acidified with an aqueous HCL
solution containing 2.1 equivalents of acid at 0°C.
The product precipatated as a white solid and was
collected by filtration. After washing with cold
water, it was dried under vacuum and recrystallized
from benzene. M.p. 115-118°C.
        For all of the compounds listed above either
fast atom bombardment mass spectrometry or elemental
analysis data was obtained confirming the structure.

                       Example 2
         Reaction of Fatty Acid Protein Modifying
                  Agents with Hemoglobin

        The reactions of compounds <u>5</u>, <u>12</u>, <u>15</u> and <u>16</u>
with native human hemoglobin (HbA) and a derivative
cross-linked between the alpha subunits (HbXL99) were
studied. In HbXL99 the cross-link bridge joins the
two lysine 99-alpha residues (Chatterjee, R. et al.
(1986) Journal of Biological Chemistry <u>261</u>, 9929-

9937; Snyder, S.R. et al. (1987) Proceedings of the
National Academy of Sciences USA 84, 7280-7284). The
reactions were carried out in 100 mM bistris buffer
at pH 7.2. The compounds were first dissolved in
dimethylsulfoxide at a concentration of 100 mM and
diluted 100-fold into the reaction mixture to give a
final concentration of 1 mM. The hemoglobin
concentration during the reaction was 0.5 mM. The
products of the reaction were identified and
quantitated by analytical isoelectric focusing
(Chatterjee, et al., 1986). In all cases the
isoelectric point of the modified hemoglobin was
decreased. For 5, 12 and 15 this is due to the
attached negatively charged group. With 16 (and
also 15) the decrease in isoelectric point is due to
the loss of the positive charge of the modified amino
group.

     Reactions of 15 and 16 with oxyHbA and
oxyHbXL99 were carried out at 37°C for 4 hours. Very
little modification of the protein was observed (less
than 5%). Substantially higher temperatures or
longer reaction times could not be studied because of
the susceptibility of oxyhemoglobin to autooxida-
tion. The reaction of 15 and 16 with deoxyHbA and
deoxyHbXL99 afforded better results. After
incubation for 4 hours at 55°C each compound gave
about 20% yield of a specific reaction product with
both native and the cross-linked hemoglobin. The
reaction with both hemoglobins was blocked by
inositol hexaphosphate (IHP). IHP binds very
tightly to deoxhemoglobin at the 2,3-diphospho-
glycerate binding site, indicating that these
compounds react with an amino group of the protein in
this region, most probably Val 1-beta or lysine 82-
beta. The two negatively charged carboxylate groups

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are sufficient to direct these reagents to react with deoxyhemoglobin at the beta cleft. The yield of the reaction can be further improved by incorporating additional negatively charged substituents into the aromatic ring.

Compounds 5 and 12, both alkylating agents, reacted selectively with deoxyHbA to yield one major product. The level of modification obtained after reaction for 6 hours at 37°C was 75% and 40%, respectively. Similar results were obtained for the reaction of these compounds with deoxyHbXL99.

The reactions of deoxyHbA with 5 and deoxyHbXL99 with 16 were performed on a larger scale to isolate the fatty acid modified derivatives for plasma half-life studies. The reactions were carried out under the same conditions as described above with 2 to 4 g of hemoglobin. The derivative modified with 16 was purified by chromatography over DEAE-Sepharose (Pharmacia). Using 0.2 M glycine pH 8.0 as the column buffer (see Chatterjee et al., 1986), the desired product was eluted with 0.1 M NaCl. The product of the reaction of deoxyHbA with 5 was isolated by preparative isoelectric focusing in a pH 6.7 to 7.7 gradient. Ampholines were obtained from Pharmacia.

                        Example 3
  Prolongation of the Intravascular Retention Time of
   Hemoglobins Modified with a Long Chain Fatty Acid

Transfusion experiments were carried out in the rat to measure the plasma half-life of the two fatty acid modified hemoglobin derivatives just described. The studies were carried out essentially as described by Snyder, S.R. et al., 1987. Briefly, hemoglobin solutions were exchanged by ultra-

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filtration into lactated Ringer's and concentrated to
7% (approximately 1 mM). Male Sprague-Dawley rats
weighing about 300 g were anesthetized with urethane
at a dosage of 1.2 g per kg body weight. A catheter
was placed in the femoral artery and 15% of the total
blood volume was removed (about 3cc) and replaced
with an equal volume of the hemoglobin solution.
Blood samples were withdrawn at subsequent times and
the plasma hemoglobin concentration was determined
spectrophotometrically.

     In a first series of experiments the
intravascular retention times of unmodified
hemoglobin and the fatty acid derivative of HbA
prepared with 5 were compared. The plasma half-life
observed for HbA was about 1.3 hours similar to that
reported previously for this model (Snyder, S.R. et
al., 1987). The half-life of the fatty acid modified
derivative was prolonged to 2.8 hours.   The plasma
half-life of hemoglobin is highly dose and species
dependent. A half-life of about 3 hours under these
conditions in the rat would translate to approxi-
mately 20 hours in man at a 50% blood volume replace-
ment.

     In a second series of experiments the
intravascular retention times of HbXL99 and HbXL99
further modified with the fatty acid derivative 16
were compared. The plasma half-life of HbXL99 was
found to be 3.2 hours as reported earlier (Snyder,
S.R., et al., 1987). Renal excretion of HbXL99
is almost completely blocked due to the
intramolecular cross-link between the alpha chains.
The plasma half-life of the fatty acid modified
derivative was increased about 2-fold to 6 hours.
This is nearly the same value observed in this model
for a hemoglobin-hemoglobin dimer cross-linked

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intermolecularly (Snyder, S.R., et al., 1987) consistent with the fact that such a dimer and the complex formed between HbXL99 and albumin have essentially the same size and molecular weight, about 130,000 daltons.

These results demonstrate that the binding of protein drugs modified with fatty acid derivatives to albumin not only inhibits their excretion by the kidneys but also slows their egress across other capillary beds. It is also apparent from these studies that in preparing fatty acid modified derivatives of hemoglobin for use as blood substitutes it is desirable to use a hemoglobin that is also cross-linked intramolecularly.

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What is claimed is:

1.

A peptide and protein drug carrier composition
which decreases metabolism and/or excretion of the
drug, said composition comprising:
a peptide/protein drug chemically linked to an apolar
bridging modifier which in turn is bound
noncovalently to albumin.

2.

The composition of claim 1 wherein the bridging
modifier is a long chain fatty acid or a derivative
thereof.

3.

The composition of claim 2 wherein the long
chain fatty acid hydrocarbon chain is from about C_{10}
to about C_{24} in length.

4.

The composition of claim 3 wherein the
hydrocarbon chain is from C_{16} to C_{20} in length.

5.

The composition of claim 1 wherein the bridging
modifier has a functional group incorporated therein
to provide an attachment means to the drug.

6.

The composition of claim 5 wherein the
functional group is selected from the group
consisting of activated carboxylic acid derivatives
including anhydrides, N-hydroxysuccinimide esters,
and phenylesters; alkylating agents such as alkyl
halides, alpha-halo carboxylic acids and amides, and
N-substituted maleimides; and carbonyl containing
agents such as aldehydes and ketones.

7.

The composition of claim 6 wherein the bridging
modifier is attached to said drug at a limited
number of sites.

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8.

The composition of claim 7 wherein the bridging
modifier is attached to hemoglobin only at the 2,3-
diphosphoglycerate binding pocket.

9.

The composition of claim 7 wherein the bridging
modifier is attached only at the SH group of
cysteine residues of the drug.

10.

The composition of claim 1 wherein the protein
drug is selected from the group consisting of
superoxide dismutase, soluble CD4 protein, insulin,
gamma-interferon, erythropoeitin, growth hormone,
tissue plasminogen activator, hemglobin and
derivatives of hemoglobin cross-linked intramolecu-
larly.

11.

A method of modifying protein and peptide drugs
so that drug retention time in the systemic system
is increased, said method comprising:

(a)   reacting a protein or peptide drug with an
      apolar bridging modifier; and thereafter,

(b)   reacting the product of step (a) with albumin
      to provide a composite of the protein or
      peptide drug linked to albumin as a carrier,
      said link being through said bridging modifier.

12.

The method of claim 11 wherein the bridging
modifier is a long chain fatty acid or a derivative
thereof.

13.

The method of claim 12 wherein the fatty acid
derived modifier has a C_{16} to C_{20} chain length.

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14.

The method of claim 11 wherein the bridging
modifier is site specifically attached to said
protein or peptide drug, as opposed to random site
attachment.

15.

The method of claim 14 wherein the protein drug
is selected from the group consisting of those
having a molecular weight below about 60,000.

16.

A method of treating patients with a protein or
peptide drug to increase systemic retention time,
said method comprising:
delivery to the systemic system of said patient a
    protein or peptide drug which is bound to
    albumin as a carrier molecule through linkage
    of both said protein or peptide drug and said
    albumin to a fatty acid derived bridging mole-
    cule.

17.

The method of claim 16 wherein the protein drug
and fatty acid derived bridging molecule are
administered to the systemic system wherein binding
to said albumin occurs endogenously.

18.

The method of claim 17 wherein the protein drug
has a molecular weight of below about 60,000.

19.

The method of claim 17 wherein the fatty acid
derived molecule has a chain length of C_{10} to C_{24}.

20.

The method of claim 19 wherein the fatty acid
derived molecule has a chain length of C_{16} to C_{20}.

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21.

The method of claim 16 wherein the duration of activity of said protein or peptide drug is controlled by administering an apolar compound which competes with said bridging molecule for binding sites on albumin and thereby terminates the action of the drug.

22.

The method of claim 21 wherein said apolar compound is a long chain fatty acid or derivative thereof.

23.

The method of claim 22 wherein the fatty acid derived molecule has a chain length of C_{16} to C_{20}.

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)*

According to International Patent Classification (IPC) or to both National Classification and IPC
Int.Cl.5   A 61 K 47/48

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category°	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	Chemical Abstracts, vol. 92, 1980, (Columbus, Ohio, US), W. Scheider:" Ligand-independent activated state of serum albumin for fatty acid binding", page 194, column 1, abstract No. 175994d, & J. Phys. Chem. 1980, 84 (8), 925-8, see abstract ---	1-15
Y	EP,A,0359428 (R.E. OFFORD) 21 March 1990, see the whole document, especially column 3, lines 18-35 ---	1-15
A	EP,A,0326618 (NIPPON HYPOX LABORATORIES INC.) 9 August 1989, see the claims ---	1-15
A	EP,A,0037388 (INSTITUT INTERNATIONAL DE PATHOLOGIE CELLULAIRE ET MOLECULAIRE) 7 October 1981, see the claims -----	1-15

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30-09-1991	07. 11. 91

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	MISS MORTENSEN

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claim numbers   16-23   because they relate to subject matter not required to be searched by this Authority, namely:

   See PCT Rule 39.1(iv):
   methods for treatment of the human or animal body
   by surgery or therapy, as well as diagnostic methods.

2. [X] Claim numbers   1-15(incompletely)   because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

   The term "apolar bridging modifier" used in claims 1 and 11
   is not a sufficiently well defined term to enable full
   understanding of the subject matter for which protection is being sought. From
   interpreration of the examples and claims this term has been understood to mean
   "a long chain fatty acid", and thus the scope of the search was restricted to
   drug-albumin conjugates containing such long chain fatty acid apolar bridging
   modifiers.See PCT Art. 6

3. [ ] Claim numbers   because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this International application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 22/10/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0359428	21-03-90	AU-A- 4190689 WO-A- 9002136 JP-T- 3501623	23-03-90 08-03-90 11-04-91
EP-A- 0326618	09-08-89	JP-A- 63215642 JP-A- 63215640 WO-A- 8806457	08-09-88 08-09-88 07-09-88
EP-A- 0037388	07-10-81	BE-A- 882541 JP-A- 57018624	16-07-80 30-01-82